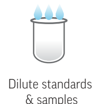
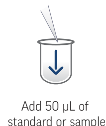
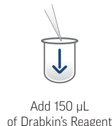
Ethos Biosciences

DRABKIN'S MICROPLATE HEMOGLOBIN ASSAY







DESCRIPTION

Drabkin's Microplate Assay is a colorimetric assay for the measurement of hemoglobin in biological samples in a convenient microplate format. It is intended for research use only, and is not intended for diagnostic purposes.

The assay depends on the reaction of Drabkin's reagent with all forms of hemoglobin except sulphaemoglobin. The product of the reaction, cyanmethemoglobin, is measured spectrophotometrically at 540 nm. Compared to the classic cuvette-based assay, Drabkin's Microplate Hemoglobin Assay uses less sample, and is amenable to high throughput. The dose-response is linear from 0.5 mg/mL to 20 mg/mL.

Hemoglobin can exist in multiple forms depending on oxygen content, carbon monoxide content, and oxidative state of the iron (ferrous, ferric, and ferryl). Each form has a unique extinction coefficient and peak absorbance wavelength. The heterogeneous nature of hemoglobin can be overcome by conversion to cyanmethemoglobin. This is achieved by reaction of hemoglobin with alkaline ferricyanide and cyanide in a single reagent. The resulting cyanmethemoglobin has a peak absorbance at 540 nm and an extinction coefficient of 0.68 for a 1 mg/mL solution.

KIT CONTENTS

- 1 x 96-well Assay Plate
- 1 x Hemoglobin Standard
- 2 x Drabkin's Reagent

Other Materials required but not provided:

- Microplate reader equipped to measure absorbance at 540 nm is required
- Adjustable pipettes and pipette tips
- Multi-channel pipettor(s) and tips for dispensing 150 µl volumes
- Tubes for preparing dilutions, i.e. microfuge tubes

Sample Collection and Storage

Blood: Venipuncture samples should be collected in tubes containing solid anticoagulants. Samples can be stored frozen for several years.

Samples: Any sample, such as column fractions, that contains visible hemoglobin can be used in the assay.

Limitations

- Certain experimental compounds or metabolites can affect results, especially those that would exhibit absorbance at 540 nm.
- Any compound that absorbs light at 540 nm will affect the assay results.
- Compounds that cause turbidity, such as lipids, abnormal plasma proteins or erythrocyte stroma, will affect the assay results. Centrifugation to clarify sample may be necessary.
- Do not mix or substitute reagents with those from other kits or sources.
- Do not use the kit beyond the expiration date on the kit label.

ASSAY PROCEDURE

This procedure describes the control, standard and sample dilutions, and their addition to the plate. Standard and sample dilutions are assayed in duplicate wells which allows analysis of up to 40 samples. Allow reagents and samples to come to room temperature before running the assay. (Note: the assay performs better when room temperature is between 20°C and 25°C.) Allow frozen samples to thaw at room temperature, and gently mix to assure homogeneity. Leave the samples undisturbed for 30-60 minutes to allow particulates to settle out.

Standard Dilutions

This procedure describes the serial dilution of hemoglobin standard.

1. Label the tubes numbers 1-7.
2. Add 120 µl of Drabkin's Reagent per tube.
3. Transfer 120 µl of Hemoglobin Standard Stock to tube 1; this is a 1:2 dilution of the standard.
4. Mix contents by aspirating and expelling the fluids 5 times.
5. Transfer 120 µl of solution from tube 1 to tube 2.
6. Mix as before.
7. Continue this procedure through tube number 7.
8. Tubes 1-7 now contain dilutions representing 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 mg/mL hemoglobin.

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Preparations of Sample Dilutions

1. Samples must be diluted to fall into the range of the assay. Optimizing the dilutions for blood samples and column fractions is necessary. Based on internal testing, a starting dilution of 1:15 for blood samples and 1:5 for column fractions in Drabkin's Reagents is suggested.
2. Performing sample dilutions in supplied microplate is not recommended.

Preparation of Drabkin's Reagent

The Drabkin's Reagent is provided as a ready-to-use solution.

Addition of Controls, Hemoglobin Standard Dilutions and Samples to the plate:

Wells A1 and A2 in the plate include assay diluent, and are intended as negative controls to standardize or "blank" the microplate reader. Do NOT use these for standards or samples.

1. Add 50 µl Drabkin's from the stock bottle to wells A1 and A2.
2. With a fresh tip, transfer 50 µl aliquots of Hemoglobin Standard Dilution Tube 1 (1:2 dilution of stock) to wells B1 and B2.
3. With a fresh tip, transfer 50 µl aliquots Hemoglobin Standard Dilution Tube 2 to wells C1 and C2.
4. Continue transferring diluted standard to the plate in this fashion, i.e. in order through H1 and H2, taking care to use a fresh tip for each new dilution.
5. Using a new tip, add 50 µl aliquots of Diluted Sample to wells A3 and A4.
6. Continue adding diluted samples to the plate, taking care to change the tip for each one.
7. The plate now contains controls, standard dilutions, and diluted experimental samples in duplicate.

Addition of Drabkin's Reagent:

1. Add 150 µl Drabkin's Reagent to each well of the plate. A multichannel pipettor is recommended.
2. Incubate the plate at room temperature for 15 minutes.

Measure the OD at 540 nm:

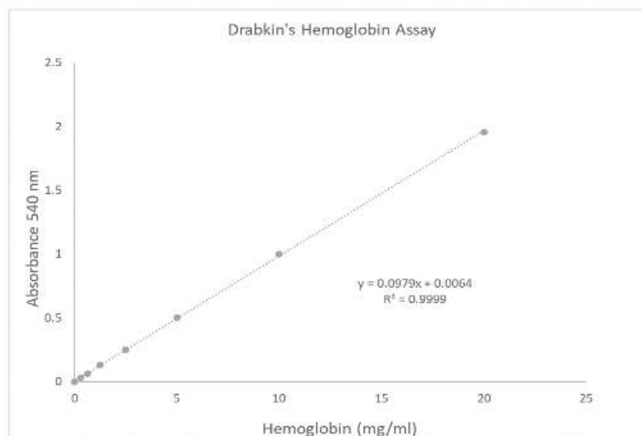
Use a plate reader to determine and record the absorbance of all wells at 540 nm, blanked against well A1.

Analysis:

Color development in the assay is proportional to the hemoglobin concentration of the diluted standard or sample. Computer-based curve-fitting software program for a linear model: fitting mean absorbance (y-axis) against the protein concentration (x-axis). The hemoglobin concentration of the samples can then be interpolated from the standard curve. Multiply the concentration by the dilution factor to determine the undilute sample concentration. Alternatively, prepare a spreadsheet entering appropriate data including standard dilution, concentration, sample dilution, and absorbance data. Determine the mean for replicate wells.

Prepare a linear plot of the standard curve:

Place the [Hemoglobin] on the x-axis and the mean absorbance on the y-axis. The data that fall into the dose-response curve constitute the usable portion of the assay.



Subject these data to linear analysis to yield a mathematical model, of the form:

$$A540 = m \cdot [Hb] + b$$

which rearranges to:

$$[Hemoglobin] = (A540 - b) / m$$

Multiply by the dilution factor of the sample to determine the concentration of undilute sample.

Quality Control:

Record Keeping: It is good laboratory practice to record the lot numbers and dates of the kit components and reagents for each assay.
Sample Handling: The samples should be secured, processed and stored as discussed above. Dilute Standard and Samples carefully. For each standard and sample, a fresh tip should be used.
Template: Record the position of each standard or sample on a microplate template.

TROUBLESHOOTING

- Color in sample well(s) is darker or lighter than highest or lowest concentrations of the standard curve. Change sample dilution protocol appropriately.
- Poor agreement between duplicate wells: This is almost always due to pipetting error. Repeat the assay.

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REFERENCES

1. Meng, F. and Alayash, A.I. (2017) Anal Biochem. 521: 11-19.
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3. International Committee for Standardization in Haematology (1967) Brit. J. Haemat. 13 (suppl.): 71

PRODUCT INFORMATION

CAT. #	DESCRIPTION
1044	Drabkin's Microplate Hemoglobin Assay

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